Identification of sika deer and red deer using partial cytochrome b and 12s ribosomal RNA genes

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Abstract: A study was conducted on the identifications of the degraded samples of sika deer (*Cervus nippon*) and red deer (*Cervus elaphus*) by phylogenetic and nucleotide distance analysis of partial Cytb and 12s rRNA genes sequences. 402 bp Cytb genes were achieved by PCR-sequencing using DNA extracted from 8 case samples, and contrasted with 27 sequences of Cytb gene downloaded from GenBank database. The values of three nucleotide distance between three suspected samples and sika deer were identical (0.026±0.006), which was smaller than the smallest nucleotide distance between eastern red deer and sika deer (0.036). Furthermore, phylogenetic analysis of sika deer and red deer indicated that the evidences located within the same cluster as sika deer. The evidences were sika deer materials. As the same way, other three suspected samples were derived from red deer. The results were further confirmed by phylogenetic and nucleotide distance analysis of 387 bp 12s rRNA gene. The method was powerful and less time-consuming and helpful to reduce the related cases with wildlife.
Keywords: Sika deer (*Cervus nippon*); Red deer (*Cervus elaphus*); Cytochrome b gene (Cytb); 12s ribosomal RNA gene (12s rRNA)
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Introduction

Sika deer (*Cervus nippon*) had a quite large population and a widespread distribution in China previously. However, because of excessive hunting and habitat loss, the survival of sika deer is seriously threatened in the wild, and only around 1 500 individuals live in the wild in China now (Guo *et al*. 2000). Endangered situation of red deer (*Cervus elaphus*) is much less serious than that of sika deer in the wild, e.g. the investigating number of red deer was near 2 189 in Dalong Mountain of Jilin Province in 1998 (Wang 2004). Sika deer and red deer are listed in Category I and II of the List of Chinese State Key Protected Wild Animals, respectively. Although captive populations of sika deer and red deer are large, product trades of sika deer and red deer are still illegal if not being authorized by government. Moreover punishment for smuggling different deer species is different according to the Wild Animal Conservation Law of China. Therefore, identification for sika deer and red deer is very important and necessary in related forensic test.

High copy number of mitochondrial DNA (mtDNA) makes it more ideal genetic marker to analyses highly degraded sample comparing with nuclear DNA. Cytb gene is of mezzo evolutional rate on mtDNA, and a small fragment can comprise genetic evolution information from inner-species to inter-species, even inter-family (Meyer *et al*. 1990). Cytb gene has been frequently used in species identification (Hsieh *et al*. 2001, 2003; M de Pancorbo *et al*. 2004; Wong *et al*. 2004). While evolutional rate of 12s rRNA gene is litter slower than that of Cytb gene, and the application of 12s rRNA gene in species identification is fewer (Girish *et al*. 2004; Yang *et al*. 2004). The aim of this paper is to identify sika deer and red deer by combining phylogenetic and nucleotide distance analysis of partial Cytb and 12s rRNA genes. This method is proved to be highly effective for degraded partial samples from sika deer and red deer, and applicable to other genetic identification of endangered species.

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Materials and methods

Samples and DNA isolation

Nine samples were provided from the laboratory of State Forestry Administration Detecting Center of Wildlife, including 5 blood samples (2 C. *n. hortulorum,* 3 *C. e. yarkandensis*) and 4 antler samples (2 C. *n. hortulorum*, 2 *C. e. xanthopygus*). Other eight samples (6 hoof tendons, 2 cartilage chips) came from a case, and genomic DNAs of samples were extracted by routine phenol-chloroform method and purified using DNA Purification Kit (Watson Biotechnologies, Inc).

PCR amplification and sequencing

Primers (L14724: GATATGAAAAACCATCGTTG and H15149: CTCAGAATGATATTTGTCCTCA) were applied to amplify partial Cytb gene from samples DNA of the case (Irwin *et al.* 1991). PCR amplification was performed in a 50 μL reaction mixture containing approximately 100 ng DNA, reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.4 μM primers, 2 mM $MgCl_2$, 0.2 mM dNTP (Takara), 2.5 units EX-Taq polymerase (Takara). Cycling condition included an initial denaturation at 94ºC for 3 min, 35 cycles of 94ºC for 30 s, 50ºC for 45 s, 72ºC for 1 min, and 1 cycle of 72ºC for 5 min (9700 Perkin-Elmer Thermal cycler).

Partial 12s rRNA gene from samples DNA of the case was amplified by primers (L1091: AAACTGGGATTAGATACC CCACTAT and H1478: GAGGGTGACGGGCGGTGTGT). Other primers (12C: AAAGCAAAGCACTGAAAATG and 12G: TTTCATCTTTCCCTTGCGGTAC) were used to amplify complete 12s rRNA gene from known samples DNA (Wang *et al.* 2000). PCR reaction mixtures and conditions of these two pairs of primer were same as primers L14724-H15149, excepted for anneal temperature were 54ºC and 52ºC, respectively. Amplified products were separated by electrophoresis on 1% agarose 1.0×TAE gel staining with ethidium bromide, and purified by Gel Extraction Mini Kit (Watson Biotechnologies, Inc). Purified products were sequenced by Bioasia Bio-technological Company (Shanghai).

Data analyses

Sequences were aligned with Clustalw Program (http://clustalw.genome.jp). The nucleotide distance and phylogenetic analysis of sequences were performed by Nucleotide Kimura 2-parameter model using Mega Computer Package (3.0 version). The phylogenetic analysis methods of Cytb and 12s rRNA genes were different for evolutional rate, namely UPGMA method (Li *et al*. 1998) and Neighbor joining method (Liu 2003), respectively. Confidence values for internal lineages were assessed with the bootstrapping option (1 000 replicates). 27 Cytb gene sequences were downloaded from GenBank database (http://www.ncbi. nlm.nih.gov), and the accession numbers were CN1304503, AY035876, AB021090–AB021093 (6 sika deer subspecies), AY044861, AY035875, AY070223, AY070224, AY044862, AY347753, AY035871, AY142327, AY142326, AY118199, AF489280, AY118198, AY244489, AY070222, AY148966, AY070221, AY044858, AY070226, AB021099, AF489281 (16 red deer subspecies), AY521063 (cow).

Results

As the case samples were highly degraded, it was difficult to amplify complete Cytb or 12s rRNA genes. Thus the partial Cytb and 12s rRNA genes were amplified by using two pairs of primers (L14724-H15149 and L1091-H1478) from samples DNA of the case. On the other hand, Cytb gene sequences of red deer or sika deer were rich in GenBank database, and 12s rRNA gene sequences of red deer or sika deer were rare in the database. Primers 12C and 12G were used to amplify complete 12s rRNA gene from the known samples. The Cytb gene and 12s rRNA gene sequences of case-1, 3, 5 were identical entirely. The 12s rRNA gene sequences of case-2 and 4 were same with the homologous sequences of *C. e. xanthopygus* absolutely. 402 bp Cytb gene, 387 bp 12s rRNA gene, and 955/956 bp complete 12s rRNA gene were submitted to GenBank database (accession numbers: DQ153244, and DQ191145–DQ191158).

It was reported that red deer consists of two distinct groups, viz. western and eastern red deer (Polziehn *et al*. 1998, 2002; Christain *et al*. 2004). Nucleotide distance analysis was performed based on above taxonomy (Table 1). Value of nucleotide distance between eastern red deer and sika deer was 0.045 (±0.009). The phylogenetic tree of sika deer and red deer was shown in Fig.1 by 402 bp Cytb gene. The result revealed three samples case-2, 4, 6 located within eastern red deer group, and three samples case-1, 3, 5 shared same group with sika deer. To further identify 6 case samples (hoof tendons), partial 12s rRNA genes were amplified and sequenced. The phylogenetic tree (Fig. 2) based on 12s rRNA gene resembled that on Cytb gene.

Discussion

The case involved two kinds of animal product, viz. hoof tendons and cartilage chip. Hoof tendons might be deer species material on morphological character, and cartilage chip was unknown due to without useful morphological character. To provide scientific and effectual evidence for the case, the method was developed combining phylogenetic and nucleotide distance analysis of partial Cytb and 12s rRNA genes to identify species. By comparing partial Cytb genes of two samples case-7, 8 (cartilage chip), we found that they had 100% homologous with that of cow, therefore two samples were defined cow material.

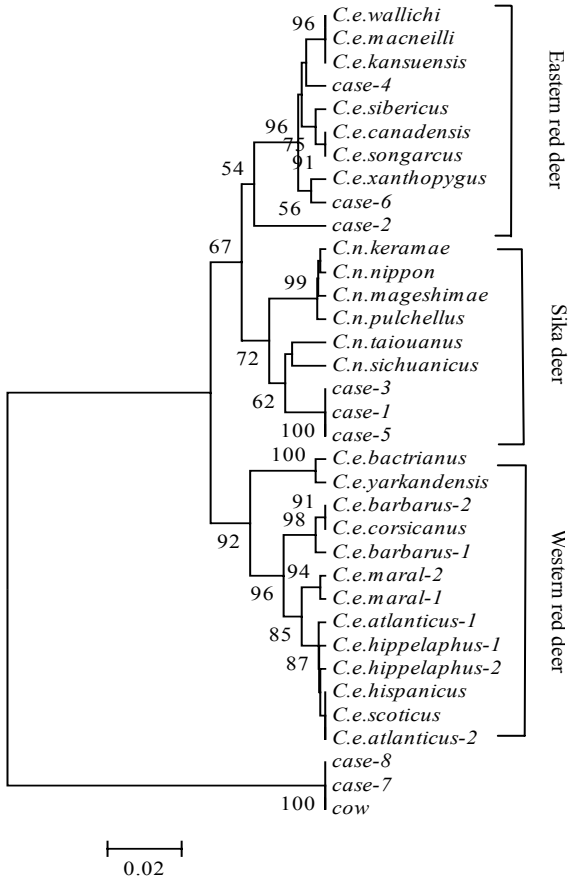

Fig. 1 Phylogenetic tree of partial of Cytb gene constructed by UPGMA method (Hiding confidence values lower than 50%)

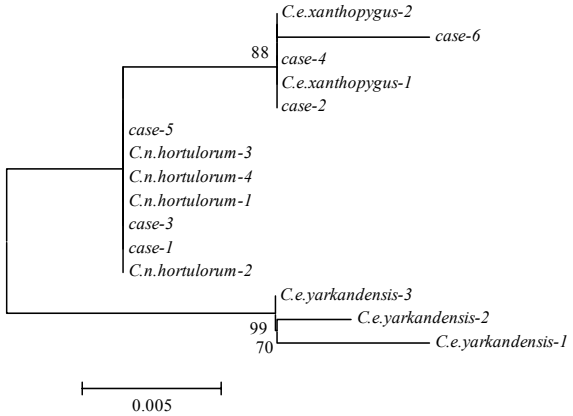

Fig. 2 Phylogenetic tree of partial 12s rRNA gene constructed by Neighbor joining method (Hiding confidence values lower than 50%)

The smallest nucleotide distance between eastern red deer and sika deer was 0.036. Values of two nucleotide distance between case- 4, 6 and eastern red deer were much smaller than 0.036, namely 0.011 (±0.004) and 0.013 (±0.004), respectively. It suggested that the case samples were most likely derived from eastern red deer. Case-2 was an exception for the value was 0.04 (±0.09). Cytb gene sequences of three samples case-1, 3, 5 were same (402 bp) absolutely, which indicated the samples came from the same species. The values of three nucleotide distance between case-1, 3, 5 and sika deer were identical (0.026±0.006),

which was smaller than 0.036. Thus, these three case samples were sika deer materials. On the other hand, the phylogenetic analysis of 402 bp Cytb gene revealed that the relationship between sika deer and eastern red deer was more closer comparing with western red deer (confidence values=67%). Three samples case-2, 4, 6 shared same group with eastern red deer, and three samples case-1, 3, 5 located within sika deer group and most close to sika deer in China. Therefore, two groups samples case-2, 4, 6 and case-1, 3, 5 were derived from red deer and sika deer, respectively, which was confirmed again by phylogenetic and nucleotide distance analysis (the data was not showed) on partial 12s rRNA gene. In a word, combining phylogenetic and nucleotide distance analysis, it was safe to presume that case samples were derived from sika deer or red deer.

Table 1. Nucleotide distance and standard error between taxa

Taxa	1	2	3	4	5	6	7	8	9	10	11
1 E-*C. e.*		0.009	0.004	0.004	0.009	0.009	0.009	0.009	0.011	0.023	0.023
2 Case-2	0.040		0.009	0.008	0.012	0.012	0.012	0.012	0.014	0.026	0.026
3 Case-6	0.013	0.038		0.004	0.009	0.010	0.010	0.010	0.011	0.022	0.022
4 Case-4	0.011	0.028	0.010		0.009	0.009	0.009	0.009	0.012	0.024	0.024
5 *C. n.*	0.045	0.072	0.047	0.047		0.006	0.006	0.006	0.010	0.022	0.022
6 Case-3	0.035	0.062	0.039	0.039	0.026		0.000	0.000	0.010	0.024	0.024
7 Case-1	0.035	0.062	0.039	0.039	0.026	0.000		0.000	0.010	0.024	0.024
8 Case-5	0.035	0.062	0.039	0.039	0.026	0.000	0.000		0.010	0.024	0.024
9 W-*C. e.*	0.064	0.092	0.062	0.067	0.057	0.055	0.055	0.055		0.023	0.023
10 Case-7, 8	0.167	0.205	0.163	0.176	0.161	0.173	0.173	0.173	0.176		0.000
11 Cow	0.167	0.205	0.163	0.176	0.161	0.173	0.173	0.173	0.176	0.000	

Notes: E-*C. e*. -- Eastern red deer, W-*C. e.*--Western red deer

Genetic divergence between sika deer and red deer was small for red deer and sika deer originated from common ancestor about one million and a half years ago (Li *et al*. 1998). It was difficult to identify sika deer from red deer when encountering partial sample (e.g. skin, bone, blood, tendon and meat) without useful morphological character. PCR-RFLP was used to differentiate red deer meat from sika deer meat (Matsunaga *et al*. 1998). Because it was complicated to apply RFLP technique, the validity of this method was not tested here. Additionally, to distinguish sika deer from red deer, a pair of universal primer of this two deer species and a pair of specific primer of sika deer were designed (Yuan *et al*. 2003). However, we found specific primers of sika deer was not always effective even when anneal temperature reached 69ºC. The reason might root in limitation of primers self, e.g. specific single nucleotide polymorphism (SNP) site did not locate at 3' end of primer. Here, using PCR-sequencing of partial Cytb and 12s rRNA genes, combining phylogenetic and nucleotide distance analysis to identify sika deer and red deer was an effectual and less time-consuming method. Furthermore, due to sequences of Cytb and 12s rRNA genes increased in public database, species identification using this method would become more and more convenient. However, this method had its shortage as other methods, and it would be failing to half-bred offspring of close relationship species owing to the mother genetic trait of mtDNA. This method would be invalidation for crossbreed offspring between sika deer and red deer, but this situation was impossible in the wild practically.

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